Circulating Tumor Cells and "Suspicious Objects" Evaluated Through CellSearch® in Metastatic Renal Cell Carcinoma

ANGELA GRADILONE[1], ROBERTO IACOVELLI[2], ENRICO CORTESI[2], CRISTINA RAIMONDI[1], WALTER GIANNI[3], CHIARA NICOLAZZO[1], ARIANNA PETRACCA[1], ANTONELLA PALAZZO[2], FLAVIA LONGO[1], LUIGI FRATI[1] and PAOLA GAZZANIGA[1]

[1]*Department of Molecular Medicine, and*
[2]*Department of Radiological, Oncological and Pathological Sciences, Sapienza University of Rome, Rome, Italy;*
[3]*Division of Geriatry, INRCA (IRCSS) Rome, Italy*

Abstract. *Background: Recent evidence supports the hypothesis that the CellSearch assay, used in the enumeration of circulating tumor cells (CTCs), may underestimate the number of CTCs, especially in tumors, such as renal cell carcinoma, frequently lacking cytokeratin expression. According to the CellSearch guidelines, all objects with no clear cytokeratin staining are defined as "suspicious objects", and are not counted as CTCs. The aim of this study was to investigate the presence of CTCs and "suspicious objects" in 25 patients affected by metastatic renal cell carcinoma (mRCC). Patients and Methods: Twenty-five patients were enrolled in the study, all with a diagnosis of metastatic clear cell RCC. The CellSearch™ system was used to count the CTC in 7.5 mL of whole blood. A further 10 ml blood obtained from each patient was used to isolate CTCs through CELLection™ Dynabeads®. The expression of cytokeratin (CK) 8, 18, 19 and CD44 were evaluated by RT-PCR. Results: Standard CTCs and suspicious objects were found in 16% and 60% of the patients, respectively. CK-8/18/19 transcripts were found in 15% and CD44 in 68% of the 19 patients with evidence of classical CTC or "suspicious objects" as assessed by Cellsearch. Conclusion: The low number of CTCs detected through CellSearch in renal cell carcinoma may be due to the presence of a CTC population with atypical characteristics and a peculiar gene expression profile, characterized by lack of cytokeratin expression and gain of CD44.*

Correspondence to: Paola Gazzaniga, Dipartimento Medicina Molecolare, Sapienza Università di Roma, Viale Regina Elena 324, 00161, Rome, Italy. Tel: +39 64451286, Fax +39 64454820, e-mail: paola.gazzaniga@uniroma1.it

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Circulating tumor cells (CTCs) are cells of epithelial origin, whose detection in the blood of metastatic breast, colorectal and prostate cancer patients represents an independent prognostic factor in terms of progression free and overall survival (1). To date, CellSearch is the only Food and Drug Administration (FDA) approved method used to obtain prognostic information through CTC count. However, recent evidence supports the hypothesis that the CellSearch technique, which relies upon the expression of epithelial cell adhesion molecule (EpCam) and cytokeratins for cellular detection, may underestimate the number of CTCs, especially those lacking cytokeratin expression due to epithelial mesenchymal transition (EMT) (2, 3). These observations support the need for improving methods to isolate cells lacking epithelial specific markers.

In a first study exploring the presence of CTCs in patients affected by all major carcinomas through CellSearch® system, a limited number of patients with metastatic renal cell carcinoma (mRCC) was included (4). The mean number of CTCs detected in the 11 mRCC patients evaluated was 1±1, compared to a significantly higher median number of CTCs detected in other cancer types, so that further investigations with the same method were not encouraged. More recently, the prognostic significance of CTCs, using immunomagnetic depletion, in RCC patients was investigated. In this study, two kinds of CTCs were detected: cytokeratin positive and cytokeratin negative (CK+, CK–), the first found only in 14% out of the 154 patients enrolled, both with a significant correlation with lymph node involvement and synchronous metastases (5). According to the CellSearch® training book, a CTC is characterized by positivity for EpCam, CK and nuclear dye; all objects with delineated nuclear image but speckled cytokeratin, as well as objects with a cytoplasm area which does not surround the nucleous, are defined as "suspicious objects", and are not counted by the operator as CTCs. To date, the significance of these cells is not clear.

In this study the presence of classically defined CTCs compared to CK– nucleous objects ("suspicious objects") was investigated through CellSearch® in a group of patients with mRCC. Furthermore, in all the patients with evidence of classical CTCs or "suspicious objects" CTCs were isolated through CELLection™ Dynabeads® and the expression of CD44, an adhesion molecule recently suggested as a marker of progression in renal cancer (6), was investigated by RT-PCR. Our interest in CD44 expression at the CTC level was also supported by the recent evidence that CD44 and EpCam function together in preparing the pre-metastatic niche (7).

The aim of the study was to investigate whether the low number of CTC reported in renal cancer through CellSearch may be due to the presence of CTC with different biological characteristics.

Materials and Methods

Twenty-five patients were enrolled in the study, all with diagnoses of metastatic clear cell RCC (age 49-71, mean age 60). All the patients were treated with first line targeted therapy. All the patients provided written informed consent. The CellSearch™ system (Veridex Corporation, Warren, NJ, USA) was used to count the CTC in 7.5 mL of whole peripheral blood. According to the CellSearch instruction book, all nucleated cells with CK+/CD45–, with the nuclear area smaller than the cytoplasmic area were defined CTCs. All the objects with speckled CK staining, or cytoplasm area not surrounding the nucleous were defined as"suspicious objects", according to the guidelines and were not counted as CTC. A further 10 ml blood draw from the selected patients was used to isolate CTCs through CELLection™ Dynabeads® (Grand Island, NY, USA) coated with the monoclonal antibody BerEp4 towards the human epithelial cell adhesion molecule EpCam, which is designed to optimally enrich bead-free, viable epithelial tumor cells. For each 10 ml of blood 250 μl CELLection™ magnetic beads coated with BerEP4 were added. Epithelial cells bind to the beads during a 30 min incubation. The enriched cells were then lysed with the Lysis Buffer supplied and 20 μl Dynabeads® Oligo(dT) 25 were added to capture mRNA. From the captured mRNA, a solid cDNA was synthesised and amplified in PCR buffer containing 25 pmol each of upstream and downstream glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers as housekeeping gene and 1.25 units of Platinum Taq polymerase (Life Technologies). In order to avoid illegitimate transcription from mononuclear cells, the cDNAs from the EpCam positive cells were routinely subjected to PCR amplifications for CD45 and CK 8/18/19 were used as markers of epithelial cells. CTCs were defined as all EpCam positive cells negative for CD45 expression but expressing at least one CK type. Each sample was then evaluated for the expression of CD44.

Results

According to the CellSearch® standard definition of CTCs (nucleated cells lacking CD45 and expressing cytokeratins) through CellSearch® CTC were found in 4/25 patients (16%, range 1-4, mean number: 1). Nevertheless, in 15/25 patients (60%) images of CD45-/ CK speckled nucleated objects, or images of CD45– objects with CK signal not surrounding the nucleous, described in the CellSearch® instruction book as "suspicious objects"were found (range 3-129, mean number: 17) (Figure 1, panel A). Due to the irregular cytokeratin signal, these images were not counted as CTCs.

In all the patients (19) with evidence of classical CTC or "suspicious objects", CTC were also eluted through CELLection™ Dynabeads®, and analysed for the expression of CK 8/18/19 and CD44. In the immunomagnetically positively selected CTCs CK-8/18/19 transcripts were found in 3/19 (15%) patients (1 patient CK8+, 2 patients CK18+) and CD44 in 13/19 (68%) (Figure 1, panel B).

Discussion

In breast cancer it has recently been suggested that the loss of epithelial antigens occurring during the EMT process may result in an underestimation of the number of CTC (2). In fact cells lacking cytokeratins are not counted through CellSearch analysis, since the classical definition criteria for CTC are not met .

This preliminary report is the first to suggest that the low number of CTCs detected through CellSearch in RCC may be due to the presence of a CTC population with atypical characteristics and a peculiar gene expression profile, mainly due to the presence of a speckled cytokeratin signal.

The low cytokeratin expression in CTCs from mRCC patients, confirmed through RT-PCR, may represent an intrinsic characteristic of the tumor, since clear cell RCC often lacks epithelial differentiation (8) This may explain the low number of CTC detected by CellSearch® in previous investigations. Consistent with this observation,the CTC of mRCC patients may be a population of EpCam+/CK– cells, which are captured through CellSearch® but not counted as CTC by the operator, due to the absence of CK specific staining.

Particularly relevant is the observation that CD44, distinctively expressed at RCC metastatic sites as compared to primary tumors (6), is highly retained by the CTCs in this tumor type. CD44, originally described as the leukocyte homing receptor, is regarded as conferring the metastatic phenotype and its role in tumor progression has been largely established in different cancer types (9, 10). Furthermore CD44 and EpCam, frequently co-expressed and up-regulated in primary tumors and metastases, have been recently suggested as markers for cancer initiating cells, in the context of preparing the pre-metastatic niche (7).

In the present series of patients, a large proportion of the EpCam selected CTC co-expressed CD44. CD44 may thus represent a new, more specific marker for the isolation of CTC in mRCC patients, allowing the detection of a growing number of CTCs in this population of patients and opening the way to a wide molecular characterization of these cells.

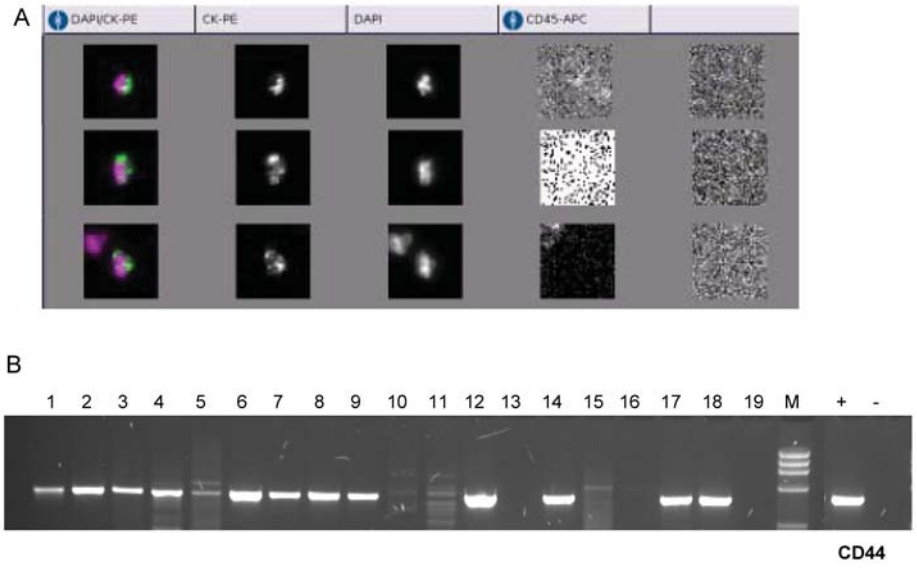

Figure 1. *Panel A: Suspicious objects detected through CellSearch in mRCC patients: images of nucleous (DAPI+) cells with CK signal not surrounding the nucleous (first lane) or speckled cytokeratin signal (second and third lane). Panel B: Expression of CD44 in 19 patients found positive for classical CTCs or suspicious objects. Lanes 1-19: CD 44 expression in CTCs from mRCC patients; M: molecular marker; +: positive control (M14 cell line); –: negative control (sample without RNA).*

Further studies on a larger patient population are needed to determine the prognostic value, if any, of the CK– DAPI+ events detected, that were not classified as CTC.

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